CORRESPONDENCE ARTICLES

A Comparative Study on PD-1 and PD-L1 Gene Expression and Concentration in Malignant and Benign Breast Tumors among Iraqi Women

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ABSTRACT

Background*: Breast cancer represents the most common form of cancer among women and is a leading cause of cancer-related deaths. Timely identification is crucial for improving therapeutic outcomes.* ***Objective:*** *This study examines the functions of Programmed cell death -1 (PD1) and Programmed death-ligand 1 (PD-L1)in the preliminary detection of breast cancer in Iraqi women. The aim of the study is to characterize the functional implications of single-nucleotide variations in programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1) in conjunction with other peripheral immune checkpoints to assess the immune competence of breast cancer patients, and to evaluate the gene and protein expression levels of immune checkpoints such as PD-1 and PD-L1 in samples obtained from patients diagnosed with breast cancer.* ***Methodology:*** *A case-control study was conducted involving 70 women diagnosed with malignant breast cancer,25 benign breast tumor and 25 healthy controls. Blood samples were collected, and immunological parameters, including PD1 and PDL1 levels, were measured using the ELISA technique, in addition molecular study to determine expression levels of PD1 and PDL1 were quantified using quantitative real-time PCR (qPCR).* ***Results****: The results showed that the ages 50-59 years, had the highest percentage of breast cancer patients. Histologically, invasive ductal carcinoma (IDC) was the most common type. BMI was associated with an increase in breast cancer risk in women. The results showed highly significant differences in PD-1 concentration between healthy subjects (1.627±0.12 aa), and patients with benign (9.246 ± 1.27 aa) and malignant (18.021±0.61 aa) tumors at a significance level of P = 0.0001**, and the LSD test (2.607) confirmed the significance of the differences between the groups. PDL_1 increased in Benign (8.615±0.32), and Malignant (12.768±0.49) breast disease patients compared with apparently healthy controls (4.339 ±0.44). PD1 and PDL1 gene expression levels were elevated in malignant breast cancer, with fold increases of 2.8 and 3.1 times, respectively, compared to benign cases.* ***Conclusion:*** *The results of the current study indicate that elevated levels of PD1 and PDL1 are closely associated with breast cancer malignancy, suggesting their potential role as biomarkers for early detection.*

INTRODUCTION

Breast cancer (BC) is the second most common malignant tumor and one of the main causes of cancer-related deaths among women worldwide. It accounts for approximately 30% of all cancer cases[1]. Women diagnosed with breast cancer comprise approximately 36% of all oncology patients. It is estimated that around 2.089 million women received a diagnosis of breast cancer[2,3] . Breast cancer comprises a variety of different malignancies that occur in the mammary glands[4]. Carcinomas are the most common type of breast cancer, while sarcomas like phyllodes tumors and angiosarcomas are rare[5]. The major hazard factor for breast cancer is being female, and its prevalence is about one hundred times more in men. Medical professionals believe that early treatment significantly improves recovery chances and reduces morbidity and mortality risks. Therefore, specialists recommend screening strategies for early diagnosis[6] .

Despite advancements in medical treatments, this condition accounts for approximately 685,000 deaths annually, representing 16% of all cancer-related fatalities among women[7]. Recent advancements in immunotherapy have shown new approaches for breast cancer treatment, primarily via modulating immune cell activities inside the tumour microenvironment tumour (MET). Tumours have the ability to circumvent immune surveillance which promote their growth and change the

microenvironment tunour to inhibit immune cell function[8] .

Protein specific as immune checkpoint regulate: initiation or stop response via activated or deactivated, serving as switches for immune functionality.*PD-1* and its ligand, *PD-L1*, are critical protein specific as immune checkpoint which negatively affect the operation and equilibrium the action of cell immune type (T cell),the last responsible for recognizing and destroying host infected cells, including carcinoma cells[9].

PD-1 is present in T-cells, while *PD-L1* is frequently located in cancer cells. The interaction between *PD-1* and its ligand *PD-L1* can trigger an inhibitory signal[10] .leading to a reduction in T-cell activity and a decrease in anti-tumor immunity. These proteins function as crucial immune checkpoint regulators that negatively influence the balance and effectiveness of T-cell immune responses[9].

METHODOLOGY

Study Design

This observational case-control study encompasses 120 women divided into three groups: 70 Iraqi women aged 23–72 years who were newly diagnosed with malignant breast cancer without a history of receiving any treatment like chemotherapy or radiotherapy and 25 women diagnosed with benign breast tumor. Samples were collected at Baquba Teaching Hospital and Al-Amal National Hospital for Oncology Treatment in Iraq, from January 2025 to June 2025.

Blood samples were obtained from these women after performing several diagnostic procedures, comprising clinical examination, ultrasound, mammogram, and multiple laboratory tests. A biopsy was conducted to confirm the diagnosis of breast cancer, In addition a control group of 25 healthy women aged matched (22–70 years) was included in the study. They did not have any tumor, fibrosis, mass, or inflammation in the breast without any surgical intervention or nipple secretions, and they were confirmed by conducting a clinical examination, ultrasound, and mammography to ensure that there was no tumor in the breast or other problems. The clinic-pathological characteristics included tumor grade and hormonal status as well as HER2 status. Verbal consent was obtained from all participants to ensure that they were fully informed of the study objectives and benefits.

Exclusion criteria: Patients with other cancer or metastasis, treated patients with chemotherapy, radiation, hormonal or other anti-cancer drugs, presence of any other autoimmune or chronic disease, taking any biological agents and recent blood transfusions (during the last 6 months).

Ethical approval

This study was conducted based on the ethical standards stipulated in the Declaration of Helsinki. Before collecting the samples, the oral and written acceptance was obtained from the patients. The study protocol and subjects' information were obtained from the local ethics committee on the basis of document number 101 dated 13/4/2025.

Sampling

Six milliliters of venous blood were collected and distributed into two parts. A volume of two milliliters of blood was introduced into a tube containing Ethylene Diamine Tetra acetic Acid (EDTA). and then take 250 whole blood to 500ul trizol , while four milliliters of blood were placed in a dry clean gel tube. The blood, in the gel tube, The sample was permitted to coagulate for a period of 15 minutes at ambient temperature, subsequently centrifuged at 2000 revolutions per minute (rpm) for 10 minutes to separate the serum. The serum was divided among four sterile, carefully sealed Eppendorf tubes and kept in a deep freezer at -80°C until it was analyzed.

Methods

***ELISA Test*:**

Determination of Human Programmed Death 1(*PD-1*) and Programmed Death 1 ligand (*PD-L1*) in patient serum was done by enzyme-linked immunosorbent assay (ELISA) The kit functions as a sandwich enzyme immunoassay designed for the in vitro quantitative assessment of *PD1* and *PDL1* .(YL Biont Catalog No: YLA0396HU)

***Molecular Study*:**

The molecular analysis included the Determine of the Expression levels of PD1 and PD-L1 using qPCR.

RNA isolation, cDNA synthesis, and Real-Time PCR

TRIzol (Life Technologies) were used for isolation of total RNA, while cDNA was prepared from RNA through Easy Script® First-Strand cDNA Synthesis SuperMix. The second section it's done by selected the cDNA sample from control and patient , two PCR tubes were used for each sample, one for gene (*PD1*) the second for (*PDL-1*). The detection of Quantity determination based on fluorescent power of Syber-GreenThe following primer sequences were used for semi-quantitative and Realtime PCR *PD-L1*, sequences for primer were obtained from Invitrogen. The sequence of PD-1, *PD-L1*, and *GAPDH* primers used in this study can be seen in Table 1.

The Power SYBR Green PCR mix (ThermoFisher Scientific) was used for real-time RT-PCR on the Step One Plus Real-Time PCR System. The thermal cycling conditions were as follows in Table 2. *GAPDH* was used as the internal control gene. ΔCt values were computed by subtracting GAPDH Ct from *PD-L1* Ct. Relative expression levels were calculated using the 2^-ΔΔCt method and using the benign group as a reference[11].

Table 1: Primers used in real-time PCR

Name	Sequence	Product size	Reference
PD1-F	TCTGTGGACTATGGGGAGCTG	198bp	Newly designed in based on NM_005018.3
PD1-R	AGAGCAGTGTCCATCCTCAGG		
PDL1-F	TGCCTTGGTGTAGCACTGACA	195bp	Newly designed in based on NM_014143.4
PDL1-R	CAGCCCCGATGAACCCCTAAA		
GAPDH-F	ATCACCATCTTCCAGGAGCGA	157bp	Newly designed in based on NM_002046.7
GAPDH-R	CAGAGGGGGCAGAGATGATGA		

Table 2: The thermal cycling conditions

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	45
Extension	60°C	30 seconds (+plate read)	
Melt Curve	60-95°C	40 minutes	1

Statistical Analysis

The Statistical Packages of Social Sciences (SPSS-26) (IBM® USA) was used to analyze and interpret data and determine the impact of various factors on study parameters expressed as frequency and percentages. The Chi-Square test of independence was used to and SD-Least Significant Difference test facilitated a meaningful comparison of these percentages, enabling a detailed examination of the relationships between the variables. The independent samples t test was used to determine the difference between continuous data which were expressed as Mean±SD. The level of significance was set as $p \leq 0.05$.

RESULTS

Table 3 represents a statistical comparison of age which shows that the most frequent age group in the malignant tumor group was 50–59 years, with 26 cases (38.99%), followed by the age group 60 years and older with 18 cases (28.99%), then the age group 40–49 years with 14 cases (19.72%), and finally the age group <30–39 years with 9 cases (12.68%). In the benign and healthy groups, the age group 50–59 years was also the most frequent, with 4 cases (40%) in each group, indicating that the majority of women with malignant tumors were over 50 years old. Regarding body mass index (BMI), it was found that the majority of those with malignant cancer were overweight (77.46%) (55 women), while the percentage of those with normal weight was 22.54% (16 women). Conversely, the obesity rate was higher in the benign tumor group (80%), indicating a difference in the pattern between the groups.

***PD-1* levels in studied groups**

The results in Table 4 compares the concentration of *PD-1* protein between the three study groups (healthy individuals, benign tumors, and malignant tumors). There were highly significant variances (P = 0.0001**) among these three groups. The least significant difference (LSD = 2.607) test also indicated that the differences between the average were significant variances present at the P≤0.01 level.

The mean *PD-1* concentration in the healthy group was 1.627 ± 0.12 ng/ml, In the benign tumor group, the mean increased to 9.246 ± 1.27 ng/ml, a significant difference from the healthy group. In contrast, the malignant tumor group had the highest mean *PD-1* concentration, 18.021 ± 0.61 ng/ml, a significant and statistically significant increase compared to the other two groups.

Table 3: Statistical Comparison of Age and Body Mass Index (BMI) Across Study Groups

Factors and level		Study group (N/%)			P- value
		Healthy group	Malignant group	Benign group	
Age groups	(<30-39)	4 (40%)	9 (12.68)	10 (50)	0.0001**
	(40-49)	0 (0%)	14 (19.72)	2(10)	
	(50-59)	4 (40%)	26 (38.99)	4(20)	
	(60≥70)	2 (20%)	18 (28.99)	4 (20)	
	Median	50	51.5	42	
Body mass index (BMI)	Over weight +obese	4 (40%)	16 (22.54)	8 (80)	0.0001 **
	Normal	6 (60%)	55 (77.46)	2 (20)	

Table 4: Average values and standard deviation of *PD1* concentration

Group of study	Mean ±SE
Healthy	1.627 ±0.12 c
Benign	9.246 ±1.27 b
Malignant	18.021 ±0.61 a
L.S.D.	2.607 **
P-value	0.0001
The different letters in same column indicate significant variances , ** (P≤0.01).	

Table 5 shows clear statistical differences in the average of *PDL-1* concentration between the three groups. The malignant tumor group recorded the highest mean concentration, 12.768 ± 0.49, followed by the benign tumor group, with a mean of 8.615 ± 0.32, while the control group recorded the lowest mean, 4.339 ± 0.44.

These differences indicate a gradual increase in *PDL-1* concentration from healthy individuals to those with benign tumors, reaching its highest levels in malignant tumors. The results showed highly significant differences at the P = 0.0001 level, with an LSD value of 1.894.

Table 5: Average values and standard deviation of *PDL-1* concentration

Group/ Type	Mean ±SE of *PDL-1*
Control	4.339 ±0.44
Benign	8.615 ±0.32
Malignant	12.768 ±0.49
L.S.D.	1.894 **
P-value	0.0001
The different letters in same column indicate significant variances , ** (P≤0.01 ** (P≤0.01).	

Molecular study:

The results of current study revealed that the mean fold of *PD1* expression (3.61±2.73) of the malignant group differed statistically significantly (p=0.048) from that of the benign group (1.02±0.24), as well as, it is found that the mean fold change of the *PDL1* of the malignant and patients groups represented by 3.83±2.31; 1.06±0.35, respectively are statistically significantly different (p=0.014) (Figure 1).

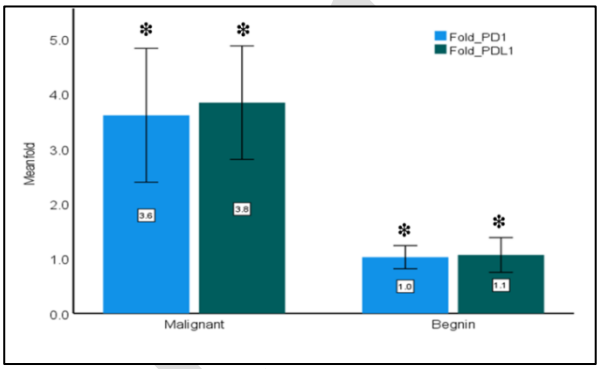

Fig. 1: Mean fold comparisons of *PD1 and PDL1*.

The results of this study indicated that the PD1 gene fold change increased by 2.82 times in the malignant group in comparison to the benign while that of the *PDL1* increased by 3.14 times in the malignant group compared to the benign (Table 6 and Table 7).

Table 6: comparison of *PD1* folding between benign malignant and groups.

Groups	Means Ct (PD1)	Means Ct (GAPDH)	ΔCt	$2^{-\Delta Ct}$	Fold
Malignant	30.072	21.1015	8.9705	0.001993	2.82
Benign	29.368	18.9	10.468	0.000706	1

Table 7: Comparison of *PDL1* folding between benign malignant and groups.

Groups	Means Ct (PDL1)	Means Ct (GAPDH)	ΔCt	$2^{-\Delta Ct}$	Fold
Malignant	30.2765	19.1515	11.125	0.000447756	3.14
Benign	31.278	18.5	12.778	0.000142377	1

DISCUSSION

Tumors are typically characterized by low immunogenicity, largely as a result of their tumor microenvironment (TME)[12]. Breast cancer is among the most common and prevalent malignant tumors impacting women. The development and occurrence of breast cancer arise from a variety of internal and external factors[13]. Unhealthy lifestyle choices, environmental factors, and social-psychological influences are all related to its progression. Studies show that five to ten percentage of breast cancer cases can be as a result of family history and genetic change via mutation, while (20%) to (30%)of breast cancer occurrences can be associated with factors that may be subject to modification[14].

In the current study, the age distribution among female breast cancer patients indicated that the most prevalent group comprised women aged 50 to 59 years (38.99%) and the smallest group was women aged <30-39 years (12.68) . Based on the current data, we

observed that malignant tumors of the breast occur mostly more than 45 years of age, while benign tumors occurred less than 45 years of age. The median age of patients with breast cancer was 51.5 years, showing a highly significant difference when compared to benign tumor patients, who had a median age of 42 years. (P< 0.001), the results of present study agree with [15.16] . while the results of the current study differ from those of Huang *et al*., who found that most cancer cases (50%) occurred among younger individuals aged 17–30 years, whereas the present study showed a higher incidence among older age groups, suggesting that age-related factors or regional variations may influence cancer incidence differently in the studied population[17].

The results of this study revealed that higher body mass index (BMI) was more predominant among all study groups, there was also significant difference in BMI among BC patients in comparison with benign breast tumor and healthy control groups. Patients with obesity are identified as having larger primary tumors associated with breast cancer and a more advanced stage of the disease at the point of diagnosis[18] . Moreover, inflammation associated with obesity facilitates not only the onset but also the advancement of breast cancer, in addition to the angiogenesis noted in the mammary tissue of most individuals who are obese and diabetic[19] . In addition Obesity causes elevation of the prevalence of breast cancer and plays important role in the development of the infection. The obesity women who develop breast cancer are more likely to present with larger tumors[20].

PD-1, a member of the CD28/CTLA-4 subfamily within the immunoglobulin (Ig) superfamily, is expressed on T cells, B cells, myeloid cells, and natural killer (NK) cells, and interacts specifically with its ligands PD-L1 and PD-L2 [21.22]. Targeting the PD-1/PD-L1 pathway has revolutionized cancer immunotherapy across several malignancies, including melanoma, head and neck squamous cell carcinoma, and non-small-cell lung cancer [17]. Upon ligand binding, PD-1 inhibits T-cell activation and cytokine secretion, leading to immune exhaustion, particularly in chronic diseases such as tumors [23]. Several studies have highlighted the prognostic relevance of PD-L1 in breast cancer, where elevated levels have been associated with reduced overall survival [24.25] . findings consistent with the results of the present study. *PD-L1* was a ligand of *PD-1*, *PD-L1* expression represents a significant biomarker for prognostication and the prediction of sensitivity to *PD-1/PD-L1* inhibitors. *PD-L1* expression predominantly occurs in tumor cells, tumor-infiltrating cells, and antigen-presenting cells (APCs) across many malignancies [26.27].

According to the current study, the concentration of *PDL-1* was elevated in patients in both Benign, and Malignant compared with apparently healthy individual , which is consistent with a study conducted by [28] and expressed disagreement with multiple studies that studied the *PD-1/PD-L1* expression status in individuals diagnosed with breast cancer, despite the findings being inconclusive our results contradict those of a previous study [29] which found the mean concentration of *PDL_1* in the serum of patients was determined to be 549.37 ng/L, whereas in the control group it was 594.22 ng/L. Notably, significant differences were observed, with a P value of 0.05.

The documented elevation of *PD1* and *PDL1* in malignant tissues, showing increases of 2.82-fold and 3.14-fold respectively, indicates a substantial function for the *PD1/PDL1* axis in tumor immune evasion within the examined cancer context [30]. The results of this study are largely consistent with recent research demonstrating that malignant breast cancer exhibits significantly increased expression of *PD-1* and its ligand, *PD-L1* [31]. This highlights its potential therapeutic target, given the association between this increased expression and aggressive tumor pattern and poorer prognosis for patients [31]. Furthermore, the current study supports the findings of a team of researchers who combined the results of 37 publications and demonstrated that observed *PD-L1* expression in studies varies widely, with expression rates ranging from 0 to 83% across subtypes [32]. In line with our research, we have demonstrated that *PD-L1* mRNA expression in blood can serve as a biomarker predicting breast cancer. The findings of this study revealed a statistically significant difference in the mRNA expression levels of *PD-L1* between benign and malignant breast cancer patients. Elevated *PD-L1* (CD274) expression in peripheral blood has been reported as a potential biomarker for breast cancer prognosis [33]. This aligns with previous evidence suggesting that increased *PD-L1* expression in tumor cells, may serve as predictive biomarkers for patients' responsiveness to immune checkpoint inhibitors [34. 35].

CONCLUSION

The results of the current study indicate that elevated levels of *PD1* and *PDL1* are closely associated with breast cancer malignancy, suggesting their potential role as biomarkers for early detection. These findings highlight the importance of integrating immune checkpoint markers into breast cancer diagnosis and prognosis.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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